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Evidence Suggesting that Virulence Maps to the P1 Region of the Coxsackievirus B4 Genome

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A chimeric virus containing the P1 region of a virulent variant of coxsackievirus B4 and the P2 and P3 regions of a nonvirulent strain was constructed from cDNA clones. The chimeric virus induced pancreatitis with concurrent hypoglycemia similar to that observed in mice infected with the virulent variant.

Coxsackieviruses, members of the *Picornaviridae*, contain a single-stranded RNA genome with positive polarity surrounded by a protein coat that comprises four capsid proteins, VP1, VP2, VP3, and VP4 (12). The genomic RNA is composed of approximately 7,400 nucleotides and contains a poly(A) tract at its 3′ terminus. A protein (VPg) is covalently attached to the 5′ terminus of the viral genome. Both viral RNA and cloned cDNA are infectious in mammalian cells (5, 10).

Coxsackieviruses of the B group have been implicated in a variety of diseases, including pancreatitis, type I insulin-dependent diabetes mellitus, myocarditis, and myositis (3, 7). These viruses are tropic for specific organ systems in both humans and animals. Variants exist within a given serotype. This diversity within a single serotype results in variability in the pathogenesis of coxsackievirus infections. Pathogenesis is further complicated by the genetic characteristics of the host (15).

To examine the contributions of both the host and the virus in the development of disease, we generated a pancreatropic variant of coxsackievirus B4 (CB4-V) and established an animal model system of pancreatitis with concurrent hypoglycemia in mice (10a). The variant, CB4-V, is highly virulent since it induces acute pancreatitis and a severe and prolonged hypoglycemia. However, the prototypical JVB strain of CB4 (CB4-P) is nonvirulent since, at the same titer, it does not induce hypoglycemia and infected mice do not succumb to infection.

Previous studies on poliovirus and Theiler's murine encephalomyelitis virus suggest that the disease phenotype maps to the P1 region of the viral genome (1, 11). Thus, to determine whether the virulent phenotype of CB4 virus mapped to the P1 region, a chimeric virus containing the P1 region of CB4-V and the P2 and P3 regions of CB4-P was constructed from cDNA clones. This approach has been successful in the generation of hybrid polioviruses (6, 8). By using RNA extracted from purified preparations of either CB4-P or CB4-V, cDNA libraries were prepared by standard methods (14).

To clone the 5′ end of the viral RNA, the technique of polymerase chain reaction was used (13). Oligonucleotide primers derived from the sequence of Jenkins et al. (4) were used to amplify the 3′ end of the cDNA product that was synthesized by reverse transcriptase with viral RNA as a template. Two sets of primers were prepared. The first pair was used to amplify cDNA corresponding to bases 1 to 1153 of the viral RNA. An *Xba*I site was added to the 5′ end primer, while an inherent *Eco*RI site was used for the 3′ end primer. The second set of primers was used to amplify cDNA corresponding to bases 1148 to 3280 of the viral RNA. For these primers, the inherent *Eco*RI and *Hin*dIII sites were used. The amplified products were subcloned into appropriately digested pBSKSM13+ (Stratagene). The map locations of some of the clones derived from CB4-P and CB4-V are shown in Fig. 1. By using the clones depicted in Fig. 1, recombinant plasmid pAAJ1, containing an entire CB4 cDNA insert, was constructed (Fig. 2). Infectious chimeric virus, herein designated CB4-V/Pa, was generated by transfecting LLC-MK2(D) cells with purified DNA from pAAJ1 by the DEAE-dextran method (2). Virus was harvested when cells exhibited 80 to 100% cytopathic effect.

Previously, we had shown that CB4-V was highly virulent in mice while CB4-P was nonvirulent (10a). Thus, to determine the phenotype of CB4-V/Pa, B10.Q mice were infected intraperitoneally with $10^{4.5}$ 50% tissue culture-infective

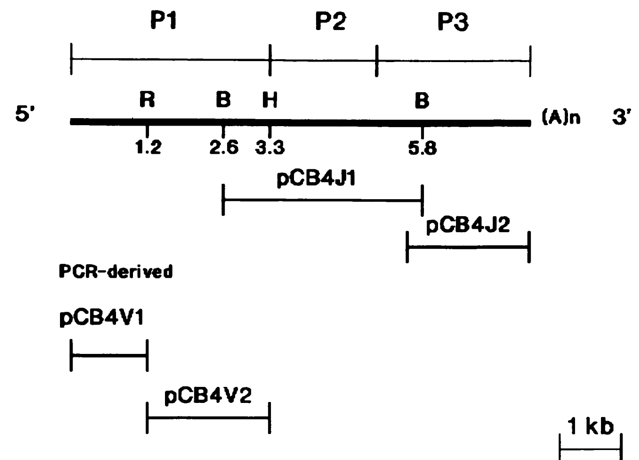

FIG. 1. Schematic representation of cDNA clones that were used to construct a chimeric CB4 virus. The top line depicts the structural organization of the CB4 viral genome (20). A partial restriction map of the CB4-P viral genome, deduced from the sequence of Jenkins et al. (4), is also shown. Both pCB4J1 and pCB4J2 were isolated from cDNA libraries of the CB4-P viral genome by standard methods. Both pCB4V1 and pCB4V2 were derived by using the polymerase chain reaction to amplify specific regions of reverse-transcribed cDNA of the CB4-V viral genome. R, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; (A)n, poly(A) tail. Numbers denote nucleotides (in kilobases).

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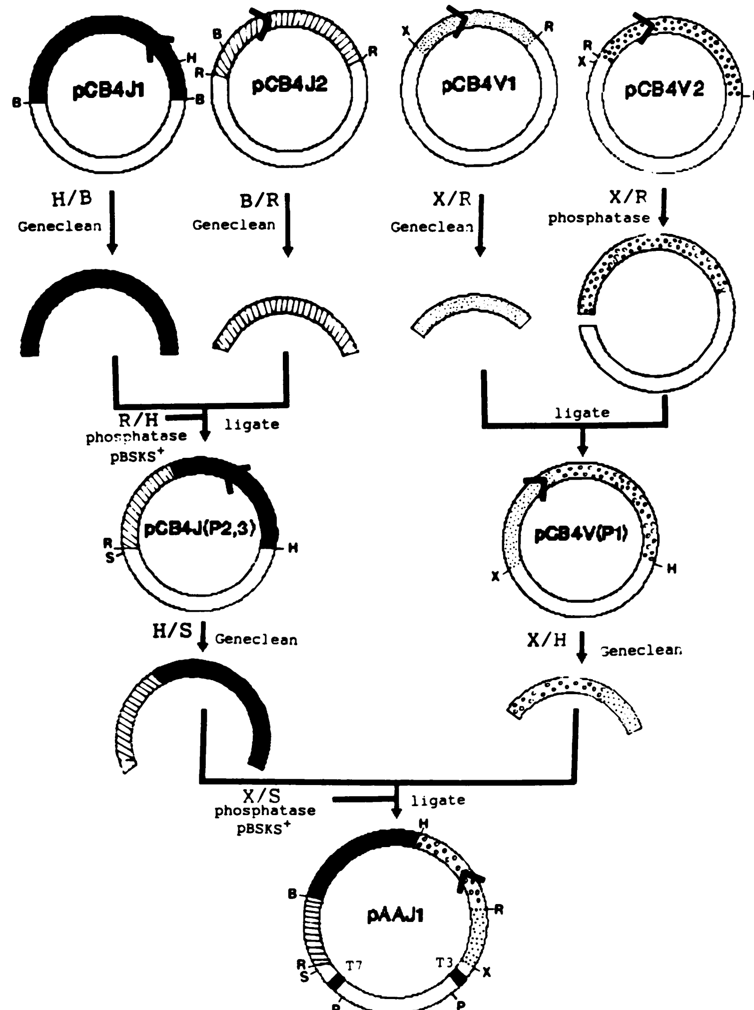

FIG. 2. Strategy for constructing a recombinant, chimeric cDNA clone, pAAJ1. The P2 and P3 regions of CB4-P were cloned from pCB4J1 and pCB4J2 to yield pCB4J(P2,3). The P1 region of CB4-V was cloned by using both pCB4V1 and pCB4V2 to yield pCB4V(P1). The chimeric recombinant, pAAJ1, was generated by using pCB4J(P2,3) and pCB4V(P1). H, *Hin*dIII; B, *Bam*HI; R, *Eco*RI; X, *Xba*I; S, *Sac*I; P, *Pvu*II. Arrows indicate direction of transcription.

doses ($TCID_{50}$) of either CB4-V, CB4-P, or the chimeric virus. Control mice were injected with phosphate-buffered saline (PBS). All animal procedures were in accord with those described in the "Guide for the Care and Use of Laboratory Animals." Pancreatic tissue was harvested at 4 days postinfection and processed for staining with hematoxylin and eosin (Fig. 3).

In mice infected with CB4-V, there was degeneration of the exocrine pancreas. A generalized degranulation of the acinar cells and partial loss of the number of exocrine secretory units was observed (Fig. 3E and F). However, the interlobular ducts and interstitial connective tissue remained intact. Furthermore, lymphocytic infiltration into localized areas of the exocrine cells was observed. Unlike CB4-V-infected mice, pancreatic tissue harvested from CB4-P-infected mice showed less pronounced changes in the exocrine tissue. Acinar cells appeared shrunken, with smaller nuclei (Fig. 3C and D). Degranulation of the acinar cells was not observed. Mice infected with the chimeric virus displayed a histopathology similar to that observed in CB4-V-infected mice (Fig. 3G and H). Since CB4-V/Pa contained the P1 region of CB4-V and the P2 and P3 regions of CB4-P, the data suggest that the virulent phenotype maps to the P1 region of the viral genome.

To determine whether the chimeric virus affected glucose concentrations in serum, nonfasted mice were bled from the tail vein, and glucose concentrations in serum were determined by the glucose oxidase method (9) prior to autopsy. Representative results are shown in Table 1. Severe hypoglycemia is defined as a 50% reduction in glucose concentration. All CB4-V-infected mice developed hypoglycemia within 4 days postinfection, while CB4-P-infected mice remained normoglycemic. Of the mice infected with CB4-V/Pa, 75% developed severe hypoglycemia. These data again suggest that the chimeric virus displayed a phenotype similar to that of CB4-V.

The P1 region of the CB4 genome comprises an untranslated region and the genes encoding the structural proteins VP1, VP2, VP3, and VP4. Thus, our data suggest that virulence is associated with either the viral structural proteins or the 5′ untranslated region (UTR). Studies on poliovirus suggest that the neurovirulent phenotype maps to the 5′ UTR (1), while studies on Theiler's murine encephalomyelitis virus suggest that the disease phenotype maps to the

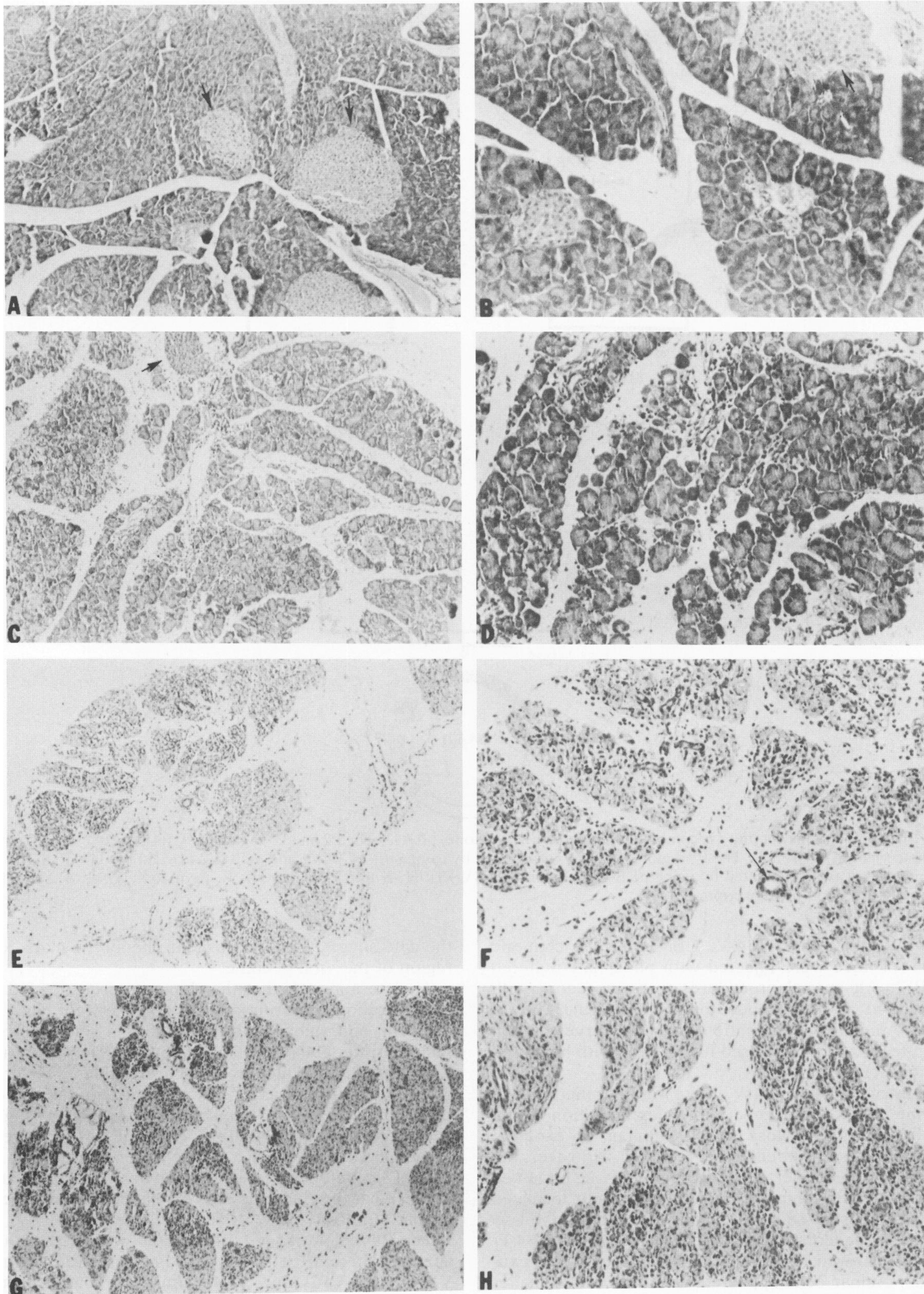

FIG. 3. Histopathology of pancreatic tissue from B10.Q mice. Mice were infected with $10^{4.5}$ $TCID_{50}$ of either CB4-P, CB4-V, or the chimeric virus. Control mice were mock-infected with PBS. (A and B) PBS-injected mice; (C and D) CB4-P-infected mice; (E and F) CB4-V-infected mice; (G and H) mice infected with the chimeric virus. Short arrow (A, B, C) denotes islet of Langerhans; long arrow (F) denotes interlobular duct. (A, C, E, G) Magnification, ×58; (B, D, F, H) magnification, ×116.

TABLE 1. Glucose concentrations in serum of B10.Q mice after infection with CB4-P, CB4-V, or the chimeric virus

Inoculum	Animal no.	Glucose concn in serum[a] (mg/dl)	
		Preinfection	4 days post-infection
PBS	1	130	132
	2	91	105
	3	98	142
CB4-P	1	169	118
	2	128	85
CB4-V	1	145	39*
	2	168	63*
	3	255	36*
Chimeric virus (pAAJ1)	1	110	38*
	2	152	71*
	3	133	38*
	4	141	121

[a] *, Hypoglycemic animal.

gene encoding VP1 (11). To determine whether the disease phenotype of CB4 virus maps to the 5′ UTR or to the genes encoding the structural proteins, additional chimeric viruses containing either the 5′ UTR of CB4-V and the structural genes and P2 and P3 regions of CB4-P or the 5′ UTR, the P2 and P3 regions of CB4-P, and the structural genes of CB4-V are being constructed. Nucleotide sequence analysis of the P1 region of CB4-V and comparison with the analogous region of CB4-P will allow localization of the bases that are important in determining virulence (Ramsingh et al., unpublished data).

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